IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Dugan et al.	**Group No.:**	1614
Serial No.:	10/083,283	**Atty. Docket No.:**	53047-31628
Filed:	February 23, 2002		
For:	***Carboxyfullerenes and Methods of Use Thereof***	**Examiner:**	Henley III, Raymond J..

DECLARATION OF DR. LAURA L. DUGAN

UNDER 37 C.F.R. §1.132

I, Dr. Laura L. Dugan, declare and state as follows:

1. All of the statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true.

2. I am the first-named inventor of U.S. Patent Application No. 10/083,283 for Carboxyfullerenes and Methods of Use Thereof filed February 23, 2002 ("Patent Application"). I am also a co-inventor on U.S. Patent 6,265,443 for Methods of Treating Neuronal Injury with Carboxyfullerene (Choi et al).

3. My current position is Professor, Larry L. Hillblom Chair in Geriatric Medicine in the Division of General Medicine and Geriatrics at the Department of Medicine, University of California, San Diego.

4. The Patent Application incorporates in it's entirety U.S. Patent 6,265,443 for Methods of Treating Neuronal Injury with Carboxyfullerene (Choi et al). Choi et al shows that $C_{60}(C(COOH)_2)_3$ compounds are effective free radical scavengers (i.e. can eliminate super-oxide radicals) and can reduce neuronal cell death in cultured cells treated with AMPA or NMDA. Choi et al also correctly states that other water soluble $C_{60}(C(COOH)_2)_n$ compounds with n=1 or n=2 are effective free radical scavengers.

5. Prior to the February 23, 2002 filing date of the Patent Application, I demonstrated that the carboxyfullerene derivatives "*C3*" ($C_{60}(C(COOH)_2)_3$; malonic acid groups at the *e,e,e* positions), "Penta-1,2" (two stereoisomers of $C_{60}(C(COOH)_2)_2(C(CHCOOH)$; groups at the *e,e,e* positions), "Tetra's" (four stereoisomers of $C_{60}(C(COOH)_2)(C(CHCOOH))_2$; groups at the *e,e,e* positions), and "*C3*-lite" (four isomers of $C_{60}(C(CHCOOH))_3$; groups are in the *e,e,e* positions) exhibited comparable superoxide dismutase SOD activity (Exhibit A1 to A3). The demonstration of superoxide dismutase activity of the " "*C3*" , "Penta", "Tetra's" and "*C3*-lite" derivatives of carboxyfullerene was accomplished using methods that are fully disclosed in the Patent Application. The "*C3*" , "Penta", and "*C3*-lite" derivatives of carboxyfullerene were also shown to reduce NMDA receptor toxicity, a form of neuronal cell death mediated by mitochondrial superoxide production (Exhibit A4 to A11) prior to the February 23, 2002 filing date of the Patent Application. The demonstration of reduced NMDA receptor toxicity of neuronal cells by the " "*C3*" , "Penta", "Tetra's" and "*C3*-lite" derivatives of carboxyfullerene was accomplished using methods that are fully disclosed in the Patent Application, which incorporates by reference US Patent No. 6,265, 443 to Choi et al. Since the carboxyfullerene derivatives "*C3*", "Penta", "Tetras" and "*C3*-lite" all exhibited SOD activity, since "*C3*", "Penta", and "*C3*-lite" all reduced NMDA receptor toxicity, and since "*C3*" was shown in the Patent Application to increase lifespan when administered to mice, one skilled in the art would have expected that all of these carboxyfullerene derivatives would also have had similar lifespan-extending capabilities as "*C3*" at comparable *in vivo* doses. It is therefore my belief that additional carboxyfullerene derivatives other than "C3" could have been used at the time of February 23, 2002 filing date of the Patent Application to obtain lifespan increases.

5. I further declare that all statements herein made by my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application.

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Dr. Laura L. Dugan

July ___, 2005

Characterization of superoxide dismutase (SOD) activity and neuroprotection against NMDA excitotoxicity for the *e,e,e* series of C60 malonic acid and acetic acid derivatives

Explanation of terms:

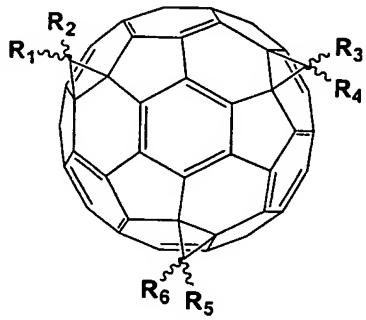
"Hexa" = C_3 ($C_{60}(C(COOH)_2)_3$ where the malonic acid groups are all at the *e,e,e* positions (***1***)

"Penta's" = $(C_{60}(C(COOH)_2)_2(C(CHCOOH))$ where the groups are at the *e,e,e* positions. There are two stereoisomers (***2***)

"Tetra's" = $(C_{60}(C(COOH)_2)(C(CHCOOH))_2$ where all groups are in the *e,e,e* positions. There are four steroisomers (***3***)

"C_3-lite" = $(C_{60}(C(CHCOOH))_3$ where all groups are in the *e,e,e* positions. There are 4 isomers.

1. $R_1=R_2=R_3=R_4=R_5=R_6=COOH$ (C_3)
2. $R_1=H$, $R_2=R_3=R_4=R_5=R_6=COOH$ (Penta Pair)
3. $R_1=R_3=H$, $R_2=R_4=R_5=R_6=COOH$ (Tetra Quartet)
4. $R_1=R_3=R_5=H$, $R_2=R_4=R_6=COOH$ (C_3-lite)
5. $R_1=R_2=COOBu$, $R_3=R_4=R_5=R_6=COOMe$
6. $R_1=R_2=R_3=R_4=COOBu$, $R_5=R_6=COOMe$
7. $R_1=R_2=COOH$, $R_3=R_4=R_5=R_6=COOMe$
8. $R_1=R_2=R_3=R_4=COOH$, $R_5=R_6=COOMe$
9. $R_1=H$, $R_2=COOH$, $R_3=R_4=R_5=R_6=COOMe$
10. $R_1=R_3=H$, $R_2=R_4=COOH$, $R_5=R_6=COOMe$



Method based on S. J. Kim et al, Biosci. Biotech. Biochem. LD946
59 (1995) 822-826.

Assay for SO peroxide scavenging:

Reagents: Tris-cacodylic acid buffer 55.6mM Tris - 55.6 c.a.
pH 8.2 - RT. med. = TCB solution

40mM Pyrogallol in 10mM HCl. 10ml dH_2O + 8.6µl 11.6 N HCl + 50.5mg pyrogallol (m.w. 126)

* Tried 2mM pyr. first → no absorbance Δ.

Reaction: 1.8ml TCB solution
.2ml Pyrogallol solution
.1ml SOD (10 U/ml) or 0.1ml H_2O
2.1ml

Vortex, pipet rapidly into cuvettes.
Kinetic protocol @ 420nm, RT, blanked against H_2O. SOD/TCB blank = H_2O absorbance at 420nm.

Plan: 2ml reaction + C_3 dose-response: Lot 99L87R
0µM, 10µM, 30µM, 100µM, 300µM, 500µM

Add as 0.1ml solutions.

Ctrl = 0.1ml TCB
10µM = x(2.5mM)(2.1ml)/(10µM) x = 0.84µl + 99µl TCB
30µM x = 2.5µl + 97µl TCB
100µM x = 8.4µl + 92µl TCB
300µM x = 25µl + 75µl TCB
500µM x = 42µl + 58µl TCB

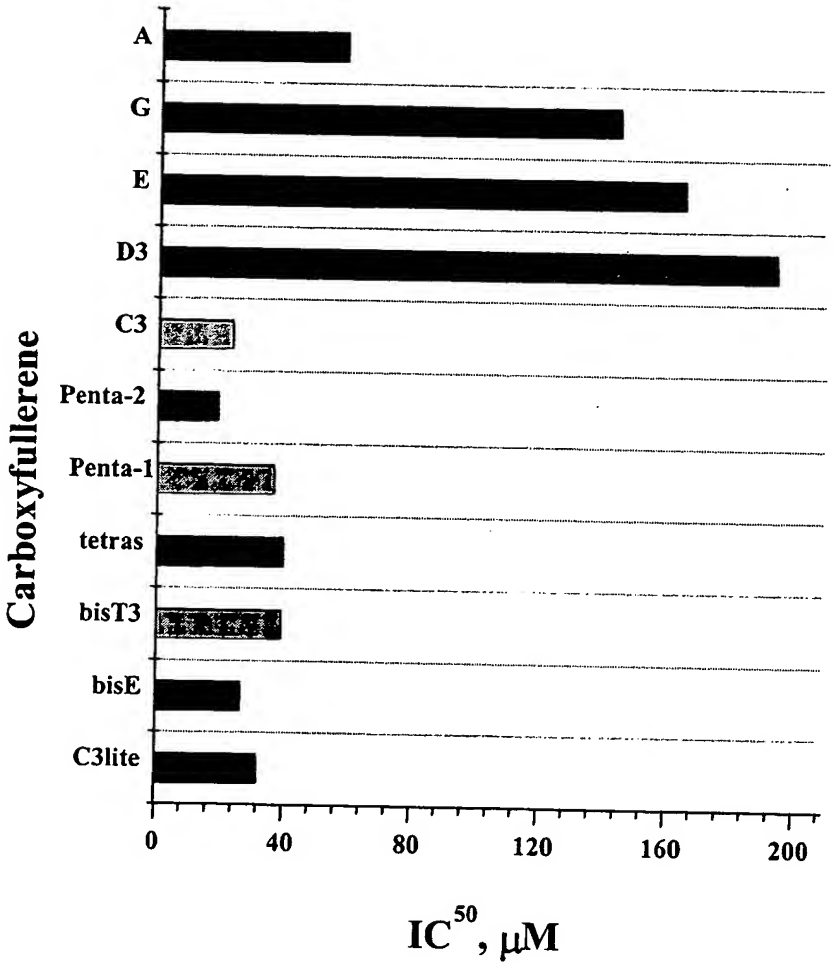
500µM → absorbance off-scale
Some ↓ SO. Try 20mM Pyr. and 10mM Pyr. @ same C_3 conc.

10mM pyr. → 2ml 40mM Py + 6ml 10mM HCl.

* need to run + SOD control again with 10mM Pyr.

EXHIBIT
A2

1/23/99 ZD


A
G
E
D3
C3
Penta-2
Penta-1
tetras
bisT3
bisE
C3lite
Carboxyfullerene
0
40
80
120
160
200
IC50, μM

and AMPA.

Plates:

Plan: NMDA 200μM x10 min
+ Hexa 10
+ " 30
+ " 100
+ Penta(1) 10
+ " 30
+ " 100
BLK

} all 3 pits each

2nd plate - Penta(2)
C_3 staff

Hexa - 99J110-1 9.368mM by UV

Penta(1) = 99J110-3 9.766mM

Penta(2) = 99J110-5 12.953mM

AMPA same plan. AMPA 10 μM

Calcs: (1.0 ml)(300 NMDA) = X 20mM
(1.0 ml)(15 Hexa or...) = X 9.368mM
" 45 = X 9.368
150

(15 Penta(1)) = X 9.766
45 " = X 9.766
150 " = X 9.766

(15 Penta(2)) = X 12.953
45 = X 12.953
150 = X 12.953

(1)(AMPA 15) = X 10mM
(1)(MK 15) = X 10mM

(1.)(10 C_3) = X 25 mM
(45) = X 25
(150) = X 25

X = 15μl NMDA
X = 1.6μl Hexa
X = 4.8μl "
X = 16μl "

X = 1.5μl Penta 1
X = 4.6μl "
X = 15μl "

X = 1.2μl Penta
X = 3.5μl "
X = 11.5μl "

X = 1.5μl AMP
X = 1.5μl MK

X = 0.6μl
X = 1.8
X = 6μl

2m @ 7:30 PM

EXHIBIT
tabbies
A4

10/15/99 XXX

Plates:

LD942

Conditions:

BLK

AMPA 8μM / 10 mL

+ (1) 0.5μM

+ " 1μM

+ 3μM

+ 10μM

Same for (3) and (5)

Calcs: 1:10 dilution of compounds → in MS

(1) Hexa = 937μ

(3) Pental = 977μ

(5) Penta2 = 1.29s

(1.25 mL)(0.75μM) = x(937) x = 1 μl

x(977) x = 0.96 μl

x(1.295) x = 0.72 μl

(1.25 mL)(1.5) = x above x = 2 μl

x = 1.92 μl

x = 1.44 μl

(1.25 mL)(4.5μM) = x above x = 6 μl

x = 5.8 μl

x = 4.3 μl

(1.25 mL)(15μM) = x above order: use basic stocks x = 2 μl

x = 1.92 μl

x = 1.44 μl

AMPA: (1.25 mL)(12μM) = x 10 mM x = 1.5 μl

MK: (1.25 mL)(15μM) = x 10 mM x = 1.875 μl

In @ 5:30 PM.

EXHIBIT

tabbies

A5

10/29/99 KD

Plates: 1%15 SAR

LD 443

Condi:

BLK

NMDA 200 µM x10 min

+ Hexa 3 µM

" 10

" 30

" 100

same for penta-1, penta-2

Calcs: (1.25 ml)(300 µM NMDA) = X 20 mM X = 18.75

(1.25 ml)(9.5) = X 937 µM X_H = 6 µl

977 µM X_{P_1} = 5.8 µl

1.295 mM X_{P_2} = 4.3 µl

(1.25)(15) = X 9368 µM X_H = 2 µl

9766 µM X_{P_1} = 1.92 µl

12,953 mM X_{P_2} = 1.44 µl

(1.25)(45) = X (9.368 mM) X_H = 6 µl

X (9.766 mM) X_{P_1} = 5.8 µl

X (12.953 mM X_{P_2} = 4.3 µl

(1.25)(150) = X same X_H = 20 µl

X same X_{P_1} = 19.2 µl

X same X_{P_2} = 14.4 µl

In @ 1330 DM

EXHIBIT

tabbies

A6

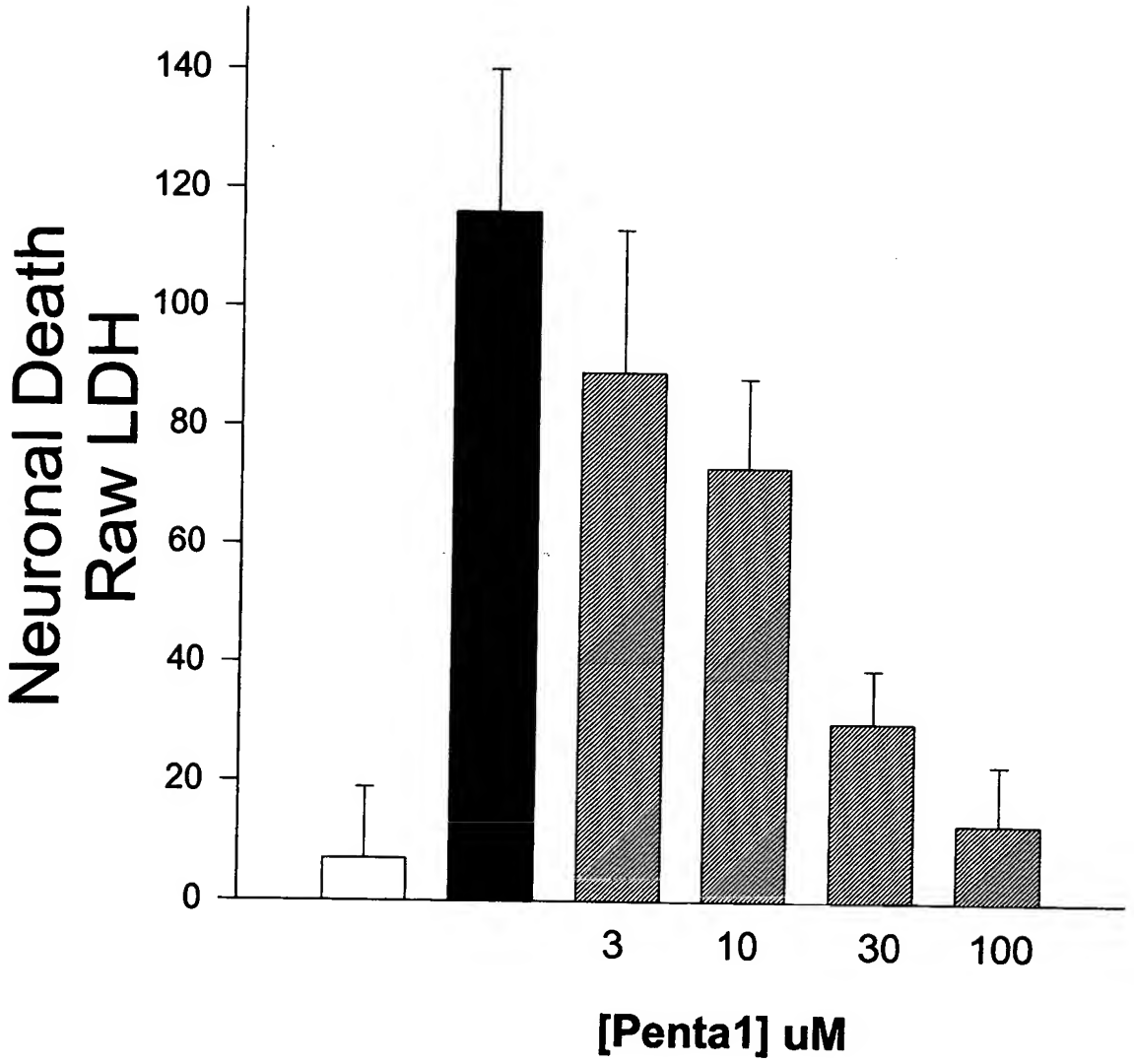
10/31/99

3 Plates! see p. 34 for calculations

for (3) Penta-1 → 100μM used now
lot "3b" 11.58 mM
x(11.58 mM) = (1.25 ml)(150μM) x = 16.2 μl

for (5) Penta-2 → 100μM used now
lot "5b" 9.43 mM
x(9.43 mM) = (1.25 ml)(150μM) x = 19.9 μl

11-14-99
NMDA 200 x 10 min
Penta-1 C_3 (3)



EXHIBIT

A8

11-14-99
NMDA 200 x 10 min
Penta-2 C_3 (5)

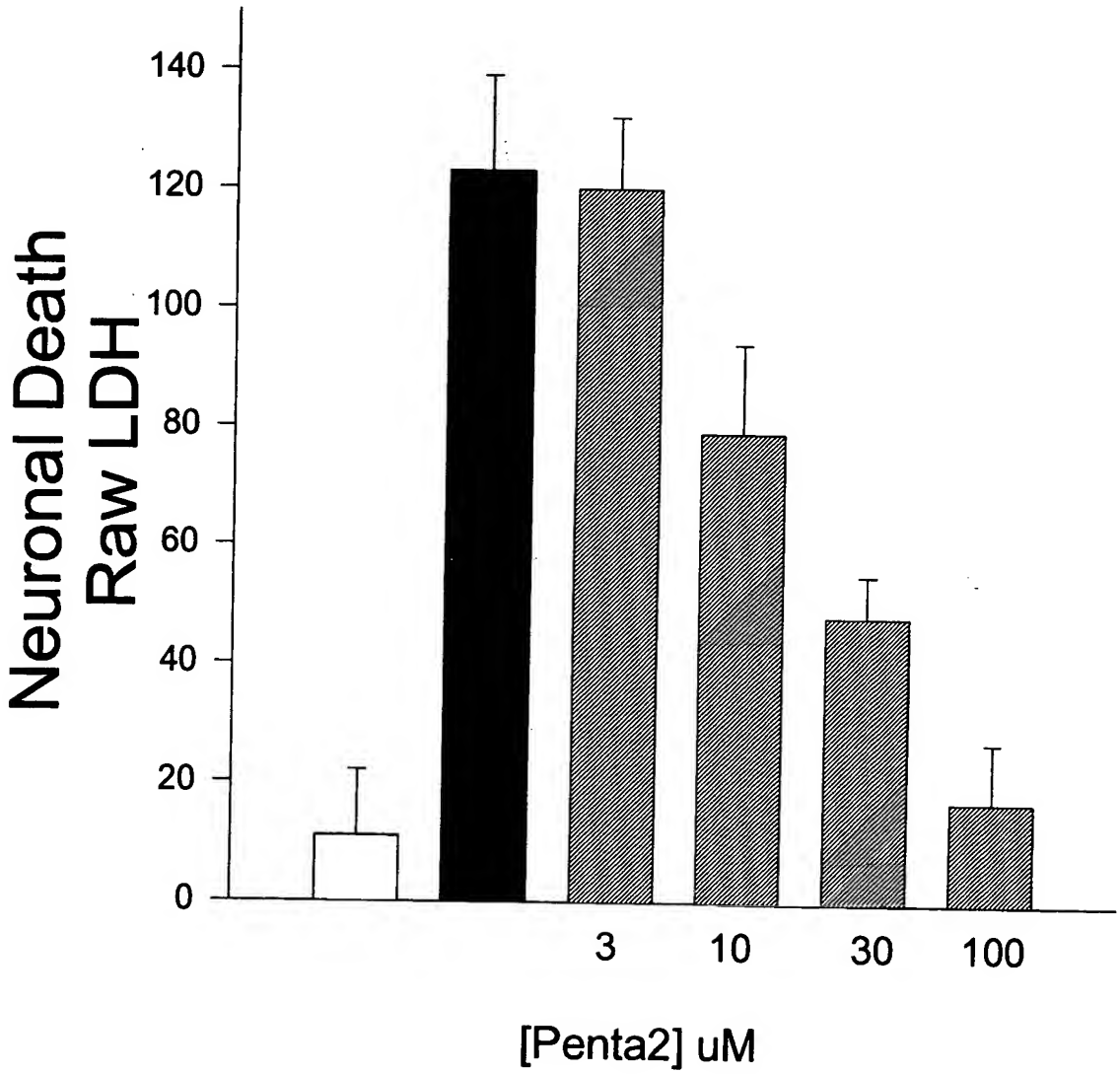


EXHIBIT
A9

PowerDesk - Z:\laura_neuron folder\C60 Projects\Structure-Function\Raw Data\LDH Toxicity as of 01-14-03\Old SPW Files

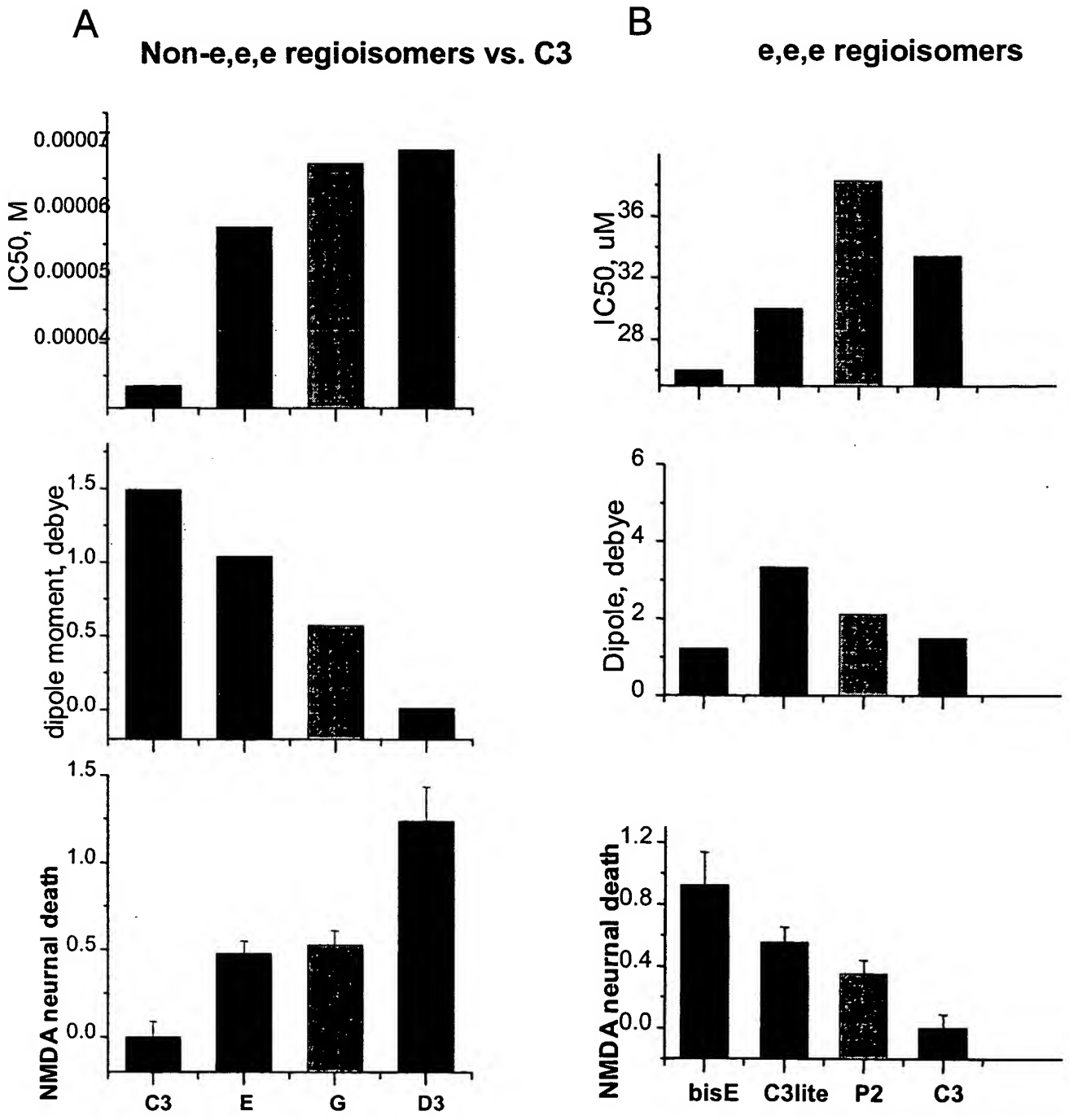
Name	Size	Date	Time	Attr	Type
HX-PTA%.SPW	22,237	11/15/1999	8:06 AM	a	SPW File
PNT1NMDA.SPW	21,294	11/14/1999	8:20 PM	a	SPW File
PNT2NMDA.SPW	21,241	11/14/1999	8:19 PM	a	SPW File

EXHIBIT

tabbies

A10

Comparison of various properties of compounds and relationship to neuroprotective efficacy. The IC50 values are for SOD activity, and show that bisE, C3, C3-lite and P2 all have very good SOD activity (note scales are different for graph in A and graph in B).



EXHIBIT

All